Electrochemical Assay for the Identification of Microorganisms

5   FIELD OF THE INVENTION

The present invention relates to assays for identifying microorganisms. More particularly, the present invention relates to testing prokaryotic and eukaryotic microorganisms in a multitest format for the rapid identification and characterization of
10   cultures important to clinical, agricultural and environmental testing.

BACKGROUND OF THE INVENTION

15   Many species of microorganisms are harmful to humans or animals, and for this reason, the correct identification of pathogens that cause disease is of great concern. Microbial diseases constitute the major cause of death in many developing countries of the world. A growing number of microbial pathogens have been identified as important food- and waterborne pathogens.
20   Traditional microbial identification methods involve a pre-enrichment step and a selective enrichment step, followed by morphologic examination, biochemical screening, growth characterization, serotyping and serological confirmation. The enrichment steps consist of growth on selective media. Morphologic examination includes observation of colony characteristics and pigmentation, microscopic examination after staining (e.g.
25   Gram stain, acid-fast stain), examination for motility and arrangement of flagella, examination for the presence of spores, capsules and inclusion bodies, and determination of the shape of the organism. Biochemical screening consists of examination for the presence or absence of constitutive enzymes or metabolic pathways in the enriched microbial culture; some biochemical tests are used generally (e.g. oxidase, nitrate
30   reduction, amino acid-degrading enzymes, fermentation and utilization of carbohydrates), while others are restricted (e.g. coagulast test for *Staphylococcus* spp.). Growth characteristics determine whether an organism is aerobic, anaerobic, facultative or microaerophilic, the specific temperature and pH conditions required for growth, the

nutrient requirements, and resistance to antibiotics. Serotyping uses antibodies to determine the type of O antigen (cell wall), H antigen (flagellar) and K antigen (capsular) present on the organism and allows both species and strain identification. Serological confirmation consists of *in vitro* reaction of patient serum (containing antibodies) with the

5   isolated organism.

Modern microbial identification methods are classified as either genotypic or phenotypic methods. Genotypic methods include all forms of analysis of microbial DNA and RNA. These methods detect genetic material present in the microorganism that, if expressed, would allow the synthesis of biochemical pathway components (e.g. for

10   metabolism, stress response factors, antibiotic resistance mechanisms).

Genotypic methods do not provide an assessment of active pathways present in a given microorganism culture at a given time. Many genotypic methods have been proposed for microbial identification and antibiotic resistance testing, including US Patent Applications 20020187490 (Tiedje et al.), 20030049636 (Bergeron et al.), 20030124545

15   (Rothman et al.), 20030180733 (Bergeron et al.), and 20040023208 (Haydock et al.).

Phenotypic identification methods assess the portion of the microbial genome that is currently expressed in a given microbial culture. These methods include traditional methods based on the observation of growth under varied conditions as well as biochemical screening for the presence of metabolic pathways. Many inventions describe

20   methods and apparati for the detection of growth and for biochemical screening in multitest formats including US Patents 3,957,586 (Babson et al.), 4,118,280 (Charles et al.), 4,129,483 (Bochner), 4,235,964 (Bochner), 4,456,380 (Kondo et al.), 4,603,108 (Bascomb), 4,604,351 (Amaral), 4,724,215 (Farber), 4,856,073 (Farber et al.), 5,089,395 (Snyder et al.), 5,164,301 (Thompson et al.), 5,223,402 (Abbas et al.), 5,340,747 (Eden),

25   5,843,699 (Strenkoski et al.), 5,888,760 Godsey et al.), 6,372,485 (Clark et al.), 6,387,651 (Bochner et al.), 6,436,631 (Bochner et al.), 6,555,322 (James et al.), and 6,611,765 (Boeufgras et al.), as well as US Patent Applications 20020064867 (Clark et al.), 20030032171 (Gemmell et al.), 20030162164 (Bochner et al.) and 20040009572 (Felice et al.) and World Patent Application WO2004050675 (Taintor). Electrochemical

30   measurements on microorganisms are described in US Patent 6,391,577 (Mikkelsen et al.) and Ertl, P. et al. (2000), *Analytical Chemistry* 72:4957-64.

Another phenotypic method has been described in which cell membrane potential changes are measured using a fluorescent dye after exposure of a culture to a bioactive chemical or biochemical in US Patent 4,343,782 (Shapiro). A database method for microbial fingerprinting by pattern recognition of pyrolysis mass spectrometric data has
5   been described in US Patent Application 20020138210 (Wilkes et al.). Another database method for microbial fingerprinting has been described in US Patent Application 20030097059 (Sorrell et al.) for matching of microbial magnetic resonance data patterns. A third database method involves capture of microorganisms on surfaces modified with specific compounds designed to recognize and bind to functional groups on the surfaces of
10 microorganisms; the binding pattern for a given organism is compared to a database of binding patterns for different organisms as described in US Patent 6,780,602 (Lloyd et al.).

Generally, no single test provides a definitive identification of an unknown microorganism. Further, currently available tests are time-consuming and often unreliable. There is a need for a rapid and effective method of identifying unknown
15 microorganisms.

SUMMARY OF THE INVENTION

20 It is an object of the present invention to obviate or mitigate at least one disadvantage of previous microorganism assays.

In one aspect of the present invention there is provided a method of identifying a microorganism comprising the steps of: obtaining a test sample of an unknown microorganism; adding a mediator or mediator mixture to the test sample in the presence
25 of an effector; assessing variation in respiration rate of the microorganism over a predetermined time period; and comparing the variation in the respiration rate of the microorganism with the variation in respiration rates of known microorganisms exposed to the effector, thereby identifying the unknown microorganism in the test sample.

In another aspect of the present invention there is provided a method of
30 differentiating Gram-positive and Gram-negative bacteria comprising the steps of: obtaining a test sample of a microorganism; adding a lipid-soluble redox-mediator to one portion of the test sample; assessing variations of respiration rate of the microorganism

over a predetermined time period; and comparing the respiration rate of this portion of the test sample with the respiration rate of another portion of the test sample not exposed to the lipid-soluble redox mediator.

Surprisingly, it has been found that respiratory rate measurements taken after
5   mixture of a test sample with an effector compound can be used for the identification and differentiation of microorganisms. The method for assessing cell responses to external stimuli comprises adding a suitable mediator or mediator mixture to a sample of the microorganism in the presence of effectors, and assessing variation of the microorganism's respiration rate over time by electrochemical measurement of mediator
10   consumption resulting from microorganism respiration. This is compared with variation of the respiration rate to other microorganism samples (stock organisms) exposed to the same effectors.

15   BRIEF DESCRIPTION OF THE FIGURES

Embodiments of the present invention will now be described, by way of example only, with reference to the attached Figures, wherein:

20   FIG. 1 shows plots of consumed charge against time for *Enterobacter aerogenes* ATCC 13048e samples from Example 1. The potential difference between the two platinum electrodes (area = 0.017 cm.sup.2) is fixed at -100 mV. Traces shown are duplicate measurements and were obtained in the presence of 10 mM (a) glucose, (b) lactic acid, (c) pyruvic acid, (d) arginine and in (e) potassium nitrate.
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FIG. 2 shows scores plots from Principal Component Analysis of respiratory activity data obtained using 22 different effector compounds in order to distinguish 10 microbial strains.

30   FIG. 3 shows scores plots from Principal Component Analysis of respiratory activity data obtained using 16 effector compounds in order to distinguish 10 microbial strains. The scores plot was generated using variances found in PC 2 and PC 4.

FIG 4 shows a 3-dimensional scores plot from Principal Component Analysis of respiratory activity data obtained using 9 effector compounds in order to distinguish 10 microbial strains.

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DETAILED DESCRIPTION OF THE INVENTION

Generally, the present invention provides a rapid new method to be used for the differentiation and identification of various microorganisms, or for the differentiation between Gram-positive and Gram-negative bacteria, or for the determination of the presence and potency of effector compounds in samples using microorganisms of known identity. More particularly, the present invention provides a method of identifying a microorganism comprising the steps of: obtaining a test sample of an unknown microorganism; adding a mediator or mediator mixture to the test sample in the presence of an effector; assessing variation in respiration rate of the microorganism over a predetermined time period; and comparing the variation in the respiration rate of the microorganism with the variation in respiration rates of known microorganisms exposed to the effector, thereby identifying the unknown microorganism in the test sample.

The present invention is based on the measurement of respiration (breathing) rates in microorganisms. Under natural conditions, microorganisms consume molecular oxygen (if they are aerobic) or another oxidant (if they are anaerobic). With aerobic organisms, oxygen is converted to water by proteins present in the membranes of the organisms. The oxygen is transformed by a process known as reduction, in which four protons and four electrons are supplied from the interior of the cell, causing the conversion of oxygen to water. Similar processes occur in anaerobic organisms, causing the reduction of oxidants such as nitrate or sulfate. These reduction processes can be made to occur with other oxidants, or mediators, that can also accept electrons from cells and transfer them to a conducting, amperometric electrode, to generate a current that is measured. The electrodes and measuring equipment used in the invention are commercially-available and well-characterized.

The present invention provides a rapid method for evaluating the effects of various compounds on microorganisms based upon the microorganism's ability to transport

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electrons to an external chemical oxidant (a mediator) that is added to the microorganism sample. The mediator interacts with the terminal components of the respiratory pathway and the extent of its consumption is related to the ability of the microorganism to respire. However, under the assay conditions described herein the extent of mediator consumption

5   is different from the microorganisms' ability to consume oxygen or another native oxidant, due to the addition of metabolizable compounds, or other compounds that affect respiratory activity, to the assay mixtures. The consumed mediator is subsequently measured electrochemically (biamperometrically or coulometrically) at the working electrode of a two-electrode electrochemical cell consisting of two polarizable platinum

10   electrodes of approximately equal surface area. This measurement can also be made using two- or three-electrode cells in which one electrode is a nonpolarizable reference electrode; a sufficiently oxidizing potential is applied to the working electrode (with respect to the reference) to reoxidize the microbially-reduced mediator. The electrochemical signals (current or charge measured as a function of time) obtained with

15   microorganism suspensions incubated in the presence of various effector compounds are significantly different. These signal differences can be used to generate a pattern that differentiates and identifies microorganisms.

The method of the present invention may further comprise a sample preparation step, in which the cell culture or suspension of microorganisms is combined with a

20   solution of the proposed effectors and incubated for a fixed time, a second step in which a mediator or mediator mixture is added, and a third step in which a biamperometric measurement is made at fixed applied potential, using standard, commercially available electrochemical instrumentation (a potentiostat) and a 2-electrode electrochemical cell. In embodiments of the present invention, the first two steps may be combined into one step.

25   In the absence of effectors, the mediator is converted by the microorganism from the oxidized to the reduced form at a rate that is characteristic of the organism and the concentrations of organism and mediator in the sample. During the measurement step, the reduced mediator is reconverted to the oxidized form at the working electrode by applying a voltage difference between the two electrodes, and the magnitude of the measured

30   current is proportional to reduced mediator concentration in the sample.

In the presence of effectors during the incubation and measurement steps, the rate of mediator reduction, and the resulting measured signals, is significantly different for

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each effector compound and organism. Depending on the chemical nature of these effectors and interaction with the target organisms, obtained respiratory activities can reach values between 10 to 600% if compared to control samples of the same organisms tested in the absence of effectors.

5   To determine the presence or absence of an effector compound in a sample a microorganism with known responses is added to the sample with a suitable mediator or mediator mixture. Variation of the microorganism's respiration rate over time is assessed by electrochemical measurement of mediator consumption. This rate is compared with variation of the respiration rate of another portion of the same microorganism not exposed

10   to the test sample.

The mediator or mediator mixture can be any suitable oxidant, including for example one or more of the following: Ferricyanide (hexacyanoferrate(III) is another name for this); Dichlorophenol-indophenol (DCIP); Ferrocene and ferrocene derivatives; Methylene blue; Janus green; Tris(bipyridyl)iron(III); the Quinone class, which includes

15   benzoquinone, naphthoquinone, menadione, anthraquinone, and substituted derivatives of these; and the Phenazine class which includes phenazine methosulfate and phenazine ethosulfate.

In another aspect of the present invention there is provided a method of differentiating Gram-positive and Gram-negative bacteria comprising the steps of:

20   obtaining a test sample of a microorganism; adding a mediator mixture containing a lipid-soluble redox mediator to the test sample; assessing variations of respiration rate of the microorganism over a predetermined time period; and comparing the respiration rate of the microorganism with the respiration rate of another sample of the same microorganism not exposed to the lipid-soluble redox mediator. This rate is compared to variations of the

25   respiratory rate of another sample not exposed to the lipid-soluble mediator compound. The omission of the lipid soluble redox-mediator compound DCIP allows the rapid distinction between cell wall properties commonly used to classify bacteria as Gram-positive and Gram-negative.

The present invention is described in the following examples.

EXAMPLE 1

Respiratory Cycle Activities in the Presence of Effector Compounds

5   In this example, the assay uses the following steps: (1) The procedure involves
combining 200 μL of the bacterial culture with 1300 μL of buffer (that contains or does
not contain the effectors and 5 μM DCIP) for a fixed time (10 min) and at a fixed
temperature (35°C) in a two-electrode electrochemical cell; (2) this is followed by the
addition of the mediator (500 μL of 0.2 M potassium ferricyanide) for a fixed time (10

10   min) and at a fixed temperature (35°C.); and (3) measurement of current at a fixed voltage
difference (-100 mV) for a fixed time (120 sec). The ferricyanide solution is added so that
the final ferricyanide conenctrations is 50 mM.
       In this configuration the assay uses two platinum electrodes each having a surface
area of approximately 0.017 cm^2. The measured current is proportional to the

15   concentration of the least-concentrated species of the redox-couple in solution. In the
present system, ferricyanide (the oxidized form) is present in great excess, leading
microorganisms to produce ferrocyanide (the reduced form) as a result of their respiratory
activity. The magnitude of the measured current indicates the quantity of the reduced
mediator present in the assay solution. The measured current is integrated over time to

20   produce a plot of total charge against time. The change in total charge is measured
between 60 and 120 seconds after the potential is applied.
       E. coli B, *Enterobacter aerogenes* ATCC 13048e, *Pseudomonas aeruginosa* and
Proteus vulgaris ATCC 6380 species were grown at 35°C over night on commercially
available MacConkey agar plates. *Bacillus cereus, Eneterococcus faecalis* and

25   *Staphylococcus aureus* ATCC 6538P species were grown at 35°C overnight on
commercially available 5%-sheep blood agar plates. Five to six colonies of each bacterial
strain were transferred into a vial containing 5.00 mL buffer solution (pH 7.2) consisting
of 4.4 g/L K_2HPO_4, 2.88 g/L KH_2PO_4, 0.48 g/ L magnesium sulphate heptahydrate, 0.048
g/L calcium chloride; 1.43 g/ L ammonium sulphate and 1.62 g/L ammonium chloride.

30   The bacterial suspensions were adjusted to a MacFarland Standard of 5.0 (5.0 x 10sup.8

cfu/ mL) or an optical density of 1.2 measured at 625 nm and cell growth was stopped by cooling the bacterial suspension for a minimum of 15 min in an ice-bath.

Traces obtained with *Enterobacter aerogenes* ATCC 13048e incubated in the absence and presence of 10 mM glucose, lactic acid, pyruvic acid, arginine and potassium

5   nitrate are shown in Figure 1. Table 1 shows the results of these measurements obtained with *Enterobacter aerogenes* ATCC 13048e and *Staphylococcus aureus* ATCC 6538P using five different effector compounds. These effectors represent a wide spectrum of known activities and mechanisms of action. Lactose represents the sole carbon source in MacConkey agar plates and consequently cultures grown on MacConkey agar plates

10 possess lactic acid pathways. Pyruvic acid is a metabolic by-product of the tricarboxylic acid cycle of organisms and is needed for the biosynthesis of the amino acids alanine, valine, leucine and glutamate, respectively. In addition, pyruvate can be used for the conversion into glucose (gluconeogenises). Amino acid fermentation (arginine) is carried out by many microbial species. All of the above substrates have been applied to identify

15 and differentiate between bacterial species using existing growth based methods. It is important to note that respiratory cycle activity measurements in the presence of these effector compounds are not the same as results obtained with traditional growth based methods or biochemical screening tests. Instead, respiratory cycle activity measurements are a reflection of the organism's ability to respire under a certain set of conditions.

20 The results shown in Table 1 have been obtained with *Enterobacter aerogenes* ATCC 13048e (Gram-negative) and *Staphylococcus aureus* ATCC 6538P (Gram-positive), which are known to utilize lactic acid as a carbon source. Obtained current signals are integrated, area normalized and relative respiratory cycle activity compared to control measurements (buffer) are plotted for each strain and effector compound.

Table 1:

Respiration assay results obtained for *Staphylococcus aureus* ATCC 6538P

and *Enterobacter aerogenes* ATCC 13048e.

Effector	conc. mM	n	*Staphylococcus aureus ATCC 6538P*			*Enterobacter aerogenes ATCC 13048e*		
			slope μC/min	normalized μC/min*mm^2	relative activity	slope μC/min	normalized μC/min*mm^2	relative activity
buffer*	-	2	9.1	5.3	100%	16.4	9.7	100%
glucose	10	2	17.9	10.6	198%	29.6	17.5	181%
lactic A	10	3	24.8	14.6	275%	27.7	16.3	169%
arginine	10	2	9.1	5.3	100%	20.5	12.1	125%
pyruvate	10	3	14.8	8.7	164%	23.4	13.7	143%
nitrate	10	2	6.9	4.1	76%	3.7	2.2	23%

* Respiratory cycle activity in buffer set as 100%-relative
acitivity

Table 2 shows results obtained with *Staphylococcus aureus* ATCC 6538P and

Enterococcus faecalis using two metabolizable effector compounds, glucose and mannose

present at 3 different concentrations. The results show an increase in measured respiratory

activities as a function of glucose or mannose concentration. It is evident that obtained

differences in respiration rates are based on effector concentrations and thus could be used

to further aid in the identification and differentiation of microorganism.

Table 2:

Slope data obtained for *S. aureus* ATCC 6538P and *E. faecalis*

Substrate (n)	Concentration	*S. aureus* μC/ min	SD	*E. faecalis* μC/ min	SD
Buffer (2)	-	7.8	± 0.1	1.2	± 0.2
Glucose (2)	1 mM	9.3	± 2.1	18.7	± 0.1
Glucose (2)	10 mM	13.9	± 1.6	18.9	± 0.1
Glucose (2)	50 mM	20.1	± 1.8	13.5	± 0.4
Buffer (2)	-	10.7	± 1.5	0.8	± 0.1
Mannose (2)	1 mM	16.6	± 0.1	17.8	± 0.7
Mannose (2)	10 mM	14.0	± 1.2	19.6	± 0.7
Mannose (2)	50 mM	15.1	± 2.1	14.5	± 2.6

EXAMPLE 2

Differentiation Between Gram-Positive and Gram-Negative Bacteria

5   The following steps are used for each test: (1) preheating for a fixed time (4 min)
and a fixed temperature (35°C) a 150 μL aliquot of buffer containing 1 mM glucose (glc)
with or without 5 μM DCIP; (2) this is followed by the addition of 50 μL of the bacterial
suspension to the sample for a fixed time (10 min) and at a fixed temperature (35°C); (3)
this is followed by the addition of the mediator (50 μL of 0.4 M potassium ferricyanide)

10   for a fixed time (10 min) and at a fixed temperature (35°C); and (4) measurement of
current at a fixed voltage difference (100 mV) between two platinum electrodes for a fixed
time (120 sec) at a fixed temperature (35°C). The final ferricyanide concentration in the
sample prior to reaction with the microorganism is 40 mM.

        In this configuration the assay uses two platinum electrodes each having a surface

15   area of approximately 0.03 cm^2. The measured current depends on the concentration of
the least-concentrated species of the redox-couple in solution. In our system, ferricyanide
(the oxidized form) is present in great excess, leading microorganisms to produce
ferrocyanide (the reduced form) as a result of their respiratory activity. The magnitude of
the measured current indicates the quantity of the reduced mediator present in the assay

20   solution. The measured current is integrated over time to produce a plot of total charge
against time. The change in total charge is measured between 30 and 120 seconds after
the potential is applied.

        E. coli B, *E. coli* Neotype, *E. coli* HB101, *E. coli* JM105, *Enterobacter aerogenes*
ATCC 13048e, *Pseudomonas aeruginosa* and *Proteus vulgaris* ATCC 6380 species are

25   grown at 35°C overnight on commercially available MacConkey agar plates.
Eneterococcus faecalis and *Staphylococcus aureus* ATCC 6538P species are grown at
35°C overnight on commercially available 5%-sheep blood agar plates. *Saccharomyces
cerevisiae* is grown at 35°C overnight on commercially available Trypton-Soy agar plates.
Five to six colonies of each bacterial strain are transferred into a vial containing 3.00 mL

30   buffer solution (pH 6.8) consisting of 4.4 g/L K_2HPO_4, 2.88 g/L KH_2PO_4, 5.00 g/ L
sodium chloride.

The omission of the lipid soluble redox-mediator compound DCIP allows the rapid distinction between cell wall properties commonly used to classify bacteria as Gram positive and Gram negative. Gram-negative bacteria contain porin proteins (channels) in their outer cell wall allowing ferricyanide to interact directly with terminal components of the respiratory pathway. Gram-positive bacteria do not possess porins and are much less reactive with ferricyanide; full respiratory activity signals are only observed if a lipid-soluble redox mediator, such as DCIP, is present. Table 3 shows a lower respiratory cycle activities obtained for Gram-positive strains after a 10 min incubation period due to the absence of the lipid soluble redox-mediator DCIP in the buffered effector solution.

Table 3:

Discrimination based on membrane compositions

Gram	Species	Condition	N	Respiratory activity $\mu C/min$	SD ±	%-Activity remaining
Positive	E. faecalis	1mM glc w/o DCIP	4	7.3	0.1	15%
		1mM glc + DCIP	4	48.2	0.5	
Positive	S. aureus	1mM glc w/o DCIP	4	13.1	0.8	39%
		1mM glc + DCIP	4	33.8	4.0	
Negative	P. vulgaris	1mM glc w/o DCIP	4	16.2	0.2	101%
		1mM glc + DCIP	4	16.1	6.1	
Negative	E. aerogenes	1mM glc w/o DCIP	4	40.1	1.7	129%
		1mM glc + DCIP	4	31.0	2.0	
Negative	E. coli B	1mM glc w/o DCIP	4	29.0	1.1	96%
		1mM glc + DCIP	4	30.1	1.4	
Negative	P. aeruginosa	1mM glc w/o DCIP	4	9.2	0.2	103%
		1mM glc + DCIP	4	8.9	0.8	

Results in Table 3 show that the appropriate choice of redox-mediators and effector compounds allow for the rapid distinction between Gram-positive and Gram-negative bacteria, which further aids in their identification. Similar results have been obtained in the presence of 1mM mannose or 1 mM succinate (instead of 1 mM glucose), where respiratory signals dropped below 60% and 80% in the absence of 5 μM DCIP,

respectively. The appropriate choice of effector compound is necessary to optimize the differences in respiratory activities for the distinction between Gram-positive and Gram-negative bacteria.

5   EXAMPLE 3

Differentiation of 10 Microbial Strains using Pattern Recognition by Principal Component Analysis of Respiratory Activity Data

In this example, each microorganism is subjected to 22 different effector

10   compounds in addition to control measurements. The following steps are used for each test: (1) preheating for a fixed time (4 min) and a fixed temperature (35°C) a 150 μL aliquot of buffer containing the effector compound and 5 mu.M DCIP; (2) this is followed by the addition of 50 μL of the bacterial suspension to the sample for a fixed time (10 min) and at a fixed temperature (35°C); (3) this is followed by the addition of the mediator (50

15   μL of 0.4 M potassium ferricyanide) for a fixed time (10 min) and at a fixed temperature (35°C.); and (4) measurement of current at a fixed voltage difference (100 mV) between two platinum electrodes for a fixed time (120 sec) at a fixed temperature (35°C.). The final ferricyanide concentration in the sample prior to reaction with the microorganism is 40 mM. For each microorganism, duplicate measurements were made of 22 different

20   effector compounds. In addition, duplicate measurements were made a control test containing no effector compound. One additional measurement was made of a control solution containing the microorganism with ferricyanide but without effector or DCIP.

In this configuration the assay uses two platinum electrodes each having a surface area of approximately 0.03 cm.^{2}. The measured current depends on the concentration of

25   the least-concentrated species of the redox-couple in solution. In our system, ferricyanide (the oxidized form) is present in great excess, leading microorganisms to produce ferrocyanide (the reduced form) as a result of their respiratory activity. The magnitude of the measured current indicates the quantity of the reduced mediator present in the assay solution. The measured current is integrated over time and normalized for electrode area to

30   produce a plot of total charge against time. The change in total charge is measured between 30 and 120 seconds after the potential is applied.

E. coli B, *E. coli* Neotype, *E. coli* HB101, *E. coli* JM105, *Enterobacter aerogenes* ATCC 13048e, *Pseudomonas aeruginosa* and *Proteus vulgaris* ATCC 6380 species are grown at 35°C over night on commercially available MacConkey agar plates. *Eneterococcus faecalis* and *Staphylococcus aureus* ATCC 6538P species are grown at

5   35°C overnight on commercially available 5%-sheep blood agar plates. *Saccharomyces cerevisiae* is grown at 35°C overnight on commercially available Trypton-Soy agar plates. Five to six colonies of each bacterial strain are transferred into a vial containing 3.00 mL buffer solution (pH 6.8) consisting of 4.4 g/L K_2HPO_4, 2.88 g/L KH_2PO_4, 5.00 g/ L sodium chloride.

10   In this example, each normalized data set containing duplicate measurements for each of 22 effector compounds is referenced to the duplicate internal controls to allow batch-to-batch comparison. The obtained signals of charge per unit electrode area for the duplicate control measurements are averaged and set at 100% respiratory cycle activity. Signals obtained for each effector compound are then averaged and compared to these

15   control samples and results are displayed as relative %-respiratory activity. The data set used to generate pattern recognition plots consists of 5 replicate batches (each batch containing duplicate measurements for each of 22 effector compounds) for each cell culture (10 strains). This is used to generate a 50 (columns) x 22 (rows) matrix. The matrix is imported into MATLAB (Version 6.0). Factor analysis is performed using the

20   MATLAB Chemometric Toolbox (Version 2.3) and involves the generation of reduced eigenvectors to determine the optimal number of factors, examination of the resulting residuals plots for randomness, and the generation of scores for the first four principle components.

TABLE 4:

Response classification by species

Conc.	Effector	P. vulgaris ATCC6380	E. faecalis	E. aerogenes ATCC13048e	E. coli JM105	E. coli HB101	E.coli B	E. coli Neotype	P. aeruginosa	S. aureus ATCC6538P	S. cerevisiae
mM		n=20	n=20	n=26	n=10	n=18	n=10	n=26	n=18	n=20	n=10
	No effector	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	+/- SD	6%	18%	4%	7%	6%	9%	14%	6%	7%	5%
3	Succinate	520%	87%	187%	287%	172%	134%	127%	102%	167%	108%
	+/- SD	70%	11%	+/- 24%	+/- 47%	28%	18%	27%	14%	46%	8%
10	L-arginine	149%	130%	129%	163%	114%	137%	114%	155%	131%	139%
	+/- SD	34%	27%	+/- 15%	19%	11%	11%	21%	19%	18%	12%
20	D-arabinose	150%	139%	106%	137%	107%	118%	103%	113%	115%	143%
	+/- SD	26%	50%	13%	16%	+/- 22%	15%	19%	20%	25%	21%
20	D-xylose	178%	128%	149%	134%	110%	102%	110%	113%	128%	116%
	+/- SD	51%	26%	26%	22%	18%	20%	20%	17%	22%	20%
10	Beta-glycerophosphate	136%	120%	131%	121%	110%	105%	108%	114%	114%	105%
	+/- SD	28%	32%	30%	37%	13%	24%	19%	21%	21%	16%
30	D-sorbitol	186%	179%	260%	168%	124%	136%	111%	104%	167%	113%
	+/- SD	33%	33%	30%	12%	29%	31%	22%	13%	24%	19%
10	Pyruvic acid	344%	136%	209%	249%	169%	120%	126%	125%	313%	109%
	+/- SD	101%	26%	38%	21%	28%	24%	30%	16%	69%	11%
20	D-lactose	162%	123%	268%	115%	192%	164%	150%	108%	138%	126%
	+/- SD	29%	20%	44%	10%	40%	21%	27%	15%	25%	21%
20	D-fructose	183%	463%	275%	202%	202%	147%	125%	136%	289%	152%
	+/- SD	48%	120%	48%	18%	39%	20%	27%	39%	106%	41%
10	Formic acid	222%	157%	124%	136%	163%	132%	114%	120%	298%	132%
	+/- SD	60%	61%	22%	34%	46%	31%	24%	22%	34%	14%
20	Citric acid	142%	117%	163%	113%	137%	161%	158%	87%	83%	103%
	+/- SD	26%	22%	49%	62%	27%	37%	36%	14%	11%	20%
20	Sucrose	164%	465%	188%	121%	110%	124%	122%	117%	290%	161%
	+/- SD	47%	114%	20%	10%	13%	10%	21%	19%	49%	16%
10	L-tryptophan	521%	232%	126%	133%	129%	98%	93%	170%	139%	188%
	+/- SD	125%	80%	22%	20%	22%	18%	19%	48%	49%	28%
10	Malonic acid	125%	146%	156%	160%	124%	141%	141%	125%	117%	110%
	+/- SD	30%	33%	28%	63%	24%	25%	32%	26%	20%	16%
5	L-ornithine	151%	138%	142%	165%	114%	135%	134%	148%	154%	137%
	+/- SD	33%	33%	17%	14%	9%	30%	23%	33%	22%	20%
10	L-lysine	219%	243%	125%	118%	125%	100%	101%	186%	159%	240%
	+/- SD	42%	46%	22%	22%	13%	26%	19%	27%	35%	42%
20	D-galactose	341%	200%	334%	173%	153%	165%	155%	130%	195%	143%
	+/- SD	101%	35%	63%	23%	31%	43%	30%	22%	55%	19%
20	D-mannose	250%	654%	296%	212%	222%	152%	139%	108%	262%	125%
	+/- SD	73%	168%	44%	41%	38%	26%	20%	14%	68%	11%
10	Alpha-ketoglutarate	201%	109%	102%	142%	116%	130%	100%	97%	115%	124%
	+/- SD	49%	25%	23%	18%	15%	37%	25%	22%	24%	17%
10	Lactic acid	457%	118%	182%	303%	171%	192%	126%	122%	622%	123%
	+/- SD	89%	33%	55%	27%	40%	49%	27%	27%	205%	19%
20	L-rhamnose	145%	105%	110%	116%	103%	119%	105%	94%	101%	124%
	+/- SD	40%	24%	33%	36%	19%	35%	25%	20%	32%	33%
10	Beta-cyclodextrin	151%	155%	110%	187%	132%	125%	81%	90%	98%	112%
	+/- SD	36%	57%	35%	42%	46%	49%	17%	21%	28%	23%

Initially, principal component analysis was applied to %-activity data shown in Table 4, in an attempt to classify responses according to species. Individual measurements (5 replicates of 10 strains) are incorporated as a 22 element column into the matrix, so that each column contained one measurement with each of the 22 effector compounds. Next,

5 all possible combinations of scores for PC 1, PC 2, PC 3 and PC4 were used in two-dimensional plots to examine groupings by species. The resulting scores plot using PC 3 and PC 4 is shown in Figure 2.

In an effort to optimize obtained pattern recognition results effector ranking followed by rank annihilation was performed. In order to assess the impact of each

10 effector compound, standard deviations of %-respiratory activities are calculated across each row in Table 4. This procedure allows for the ranking of individual effectors to the importance of their contributions to variances found between 10 organisms.

Table 5:

15 Effector ranking according to found variances between 10 strains

Rank	Effector	± SD
1	Lactic acid	171%
2	D-mannose	158%
3	Succinate	130%
4	L-tryptophan	126%
5	Sucrose	112%
6	D-fructose	103%
7	Pyruvic acid	86%
8	D-galactose	76%
9	Formic acid	58%
10	L-lysine	56%
11	D-sorbitol	48%
12	D-lactose	48%
13	Beta-cyclodextrin	33%
14	Alpha-ketoglutarate	31%
15	Citric acid	30%
16	D-xylose	23%
17	D-arabinose	17%
18	Malonic acid	17%
19	L-arginine	16%
20	L-rhamnose	15%
21	L-ornithine	14%
22	Beta-glycerophosphate	11%

Higher standard deviation values for individual effector compounds are considered indicative of the greater importance of that effector for the grouping of organisms in the scores plot. Effectors (rows) are then cumulatively eliminated from the data matrix, beginning with beta-glycerophosphate and continuing upwards from the bottom of

5   Table 5.

Figure 3 shows the scores plot of PC 3 and PC 4 obtained from the reduced matrix using the first 16 effector compounds. Additionally, *E. coli* subspecies (the two wild type strains *E. coli* B and *E. coli* Neotype, and the two laboratory strains *E. coli* JM105 and *E. coli* HB101) form identifiable subpopulations that are clustered in close proximity to each

10   other with respect to the clusters representing other species.

Further reduction of the matrix to 9 effector compounds is possible using a 3-D projection of PC 2, PC3, PC 4 as shown in Figure 4. This illustrates that the assay method of the present invention can be used for microorganism identification using more complex mathematical methods.

15

EXAMPLE 4

Probability Method for the Identification of Unknown Microbial Samples

20   In this example, experiments were conducted that were identical to Example 3, and data shown in Table 4 were used to establish a reference database for the ten strains of microorganisms. In the reference database, ten individual measurements for each effector compound with each microorganism allow the establishment of a mean respiratory activity value along with its confidence interval. A batch experiment (described in Example 3)

25   conducted on an unknown organism yields two measurements per effector compound. These two values are averaged, and the average is compared to the 90% confidence intervals for the same effector for each organism in the reference database. An "effector match" is indicated for that effector when the measurement for the unknown organism falls within the confidence interval for the same effector compound with one organism in

30   the reference database. The "overall match" is calculated simply as the number of effector matches to a given organism divided by the total number of effectors (twenty-two).

Table 6 shows the results obtained from a comparison of three replicate measurements of 10 unknown samples against the established library. High percentage match values indicate that the unknown culture provides essentially the same pattern of respiratory response to effector compounds as a known sample from the reference

5   database. It can be seen in Table 6 that the highest percentage match values for each of the 30 unknowns are obtained with the database entries corresponding to the same organisms.

These results demonstrate that patterns of electrochemically-measured respiratory activity responses to effector compounds can be used for the identification of unknown

10   microorganisms.

Table 6: Identification results obtained using statistical analysis method by comparison of unknown sample to reference library

Unknown	Prediction	% Match		
		1	2	3
E. faecalis	E. faecalis	96%	100%	96%
	S. cerevisiae	72%	76%	68%
	E. coli B	68%	68%	64%
E. aerogenes	E. aerogenes	84%	92%	88%
	E. coli B	64%	76%	72%
	S. aureus	60%	60%	68%
P. vulgaris	P. vulgaris	88 %	88%	92%
	E. coli B	64 %	60%	56%
	S. aureus	60 %	48%	80%
S. aureus	S. aureus	92%	92%	88%
	E. faecalis	80%	84%	80%
	P. vulgaris	76%	76%	76%
P. aeruginosa	P. aeruginosa	96%	92%	92%
	S. cerevisiae	88%	88%	80%
	E. faecalis	80%	76%	80%
E. coli B	E. coli B	88%	92%	88%
	E. coli Neotype	88%	76%	88%
	E. coli HB101	84%	76%	76%
E. coli HB101	E. coli HB101	100%	84%	76%
	E. coli B	88%	84%	72%
	E. coli Neotype	80%	60%	72%
E. coli Neotype	E. coli Neotype	88%	100%	96%
	E. coli B	80%	92%	92%
	E. coli HB101	64%	80%	84%
E. coli JM105	E. coli JM105	92%	84%	80%
	S. aureus	80%	72%	72%
	P. vulgaris	76%	64%	72%
S. cerevisisae	S. cerevisiae	96%	92%	88%
	P. aeruginosa	88%	80%	88%
	E. coli B	84%	80%	80%

The above examples are illustrative only. It is not intended that the invention be limited to the above examples. Many variations will be apparent to those who are knowledgeable in the field, and such variations are within the scope of the invention as described and claimed, whether or not expressly mentioned herein.